

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A23L 3/3571, A23C 19/032 C12N 11/02, A23C 19/06	A1	(11) International Publication Number: WO 92/22221 (43) International Publication Date: 23 December 1992 (23.12.92)
(21) International Application Number: PCT/NL92/00104 (22) International Filing Date: 11 June 1992 (11.06.92) (30) Priority data: 91201442.0 11 June 1991 (11.06.91) EP (34) Countries for which the regional or international application was filed: NL et al. (71) Applicant (for all designated States except US): GIST-BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). (72) Inventors; and (75) Inventors/Applicants (for US only): DE JONG, Sylvia, Josefine [NL/NL]; Van der Haertstraat 66, NL-2613 ZC Delft (NL). DE HAAN, Ben, Rudolf [NL/NL]; Wilgendreef 95, NL-2272 EN Voorburg (NL). TAN, Hong, Sheng [NL/NL]; Buizerdstraat 94, NL-2665 TG Bleiswijk (NL).	(74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents and Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL). (81) Designated States: AU, CA, JP, US. Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: LONG-TERM ANTIMICROBIAL ACTIVITY OBTAINED BY SUSTAINED RELEASE OF HYDROGEN PEROXIDE		
(57) Abstract The present invention discloses methods and means for obtaining long-term antimicrobial activity of the lactoperoxidase system. This long-term activity of the LP system is obtained by the sustained release of hydrogen peroxide. Sustained release is obtained by immobilisation of components of the LP system. Furthermore applications of this system in the conservation of food-stuffs are disclosed.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	RO	Romania
CA	Canada	IT	Italy	RU	Russian Federation
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

5 LONGTERM ANTIMICROBIAL ACTIVITY OBTAINED BY SUSTAINED
 RELEASE OF HYDROGEN PEROXIDE

Technical field

10 The present invention relates to an improvement in the
 use of an antimicrobial system. Specifically, the invention
 relates to the sustained release of hydrogen peroxide. The
 hydrogen peroxide produced may be used as such or it may be
 used in combination with suitable reactants to produce
15 substances with antimicrobial activity, specifically
 hypothiocyanate.

Background of the invention

20 Microbial contamination of food and feed can cause
 severe health problems. Recent examples are the different
 outbreaks of human listeriosis that have been reported in
 Canada (Schlech et al. 1983. N.Engl.J.Med. 308 : 203-206), in
25 the United States (Fleming et al. 1985. N.Engl.J.Med. 312 :
 404-407 and Linnan et al. 1988. N.Engl.J.Med. 319 : 823-828)
 and in Switzerland (Food Chem. News. 1987. Dec, 7).

 Microbial contamination can also adversely affect
 products containing proteins or other microbially degradable
30 components.

 Different methods to prevent microbial contamination of
 susceptible products are known such as, chemical methods
 (addition of compounds such as sulphite, nitrite, benzoic
35 acid, sorbic acid) and the use of bacteriocins. Due to the
 suspected and proven side-effects of the chemicals used in
 the chemical methods, the acceptability of such methods is
 becoming more and more questionable. Furthermore, the
 applicability of bacteriocins is restricted due to the

relatively high specificity of these molecules toward specific microorganisms. This would necessitate the use of mixtures of many different bacteriocins in order to be effective against microorganisms.

5

The disadvantages of the above-mentioned methods stimulated the search for more acceptable methods. One way to avoid the above problems is the use of naturally occurring antimicrobial systems. Turning the attention to natural
10 mechanisms for preventing microbial growth, an antimicrobial system in milk was identified to be the so-called lactoperoxidase system (LP system). The use of this lactoperoxidase system, which has a broad range of applicability, is of increasing importance.

15 The lactoperoxidase/thiocyanate/hydrogen peroxide system is an antimicrobial system which is indigenous to the major body fluids such as raw milk, tears and saliva.

The properties of this system have been reviewed by Reiter and Harnülv (1984. J. Food Protect. 47 : 724-732) and
20 Pruitt and Reiter (1985. In 'The lactoperoxidase system chemistry and biological significance' Eds. Pruitt, K.M. and Tenovuo, D. p. 144-178 New York , Marcel Dekker, Inc.).

Schematically, the lactoperoxidase system can be
25 represented by a three-step process;

- a) the hydrogen peroxide production step; the reaction of an oxidoreductase with an oxidizable substrate with the concomitant production of hydrogen peroxide,
- b) the lactoperoxidase reaction step; in this step
30 thiocyanate is converted to hypothiocyanate by reaction with hydrogen peroxide which reaction is catalyzed by lactoperoxidase,
- c) the antimicrobial reaction; wherein hypothiocyanate inactivates the microorganisms.

35

Instead of in situ production of the hydrogen peroxide, hydrogen peroxide can also be slowly added to the mixture to

- 3 -

be protected. Furthermore, it is possible to use soluble inorganic peroxides from which peroxide is gradually released. For practical reasons however it is better to generate the hydrogen peroxide in situ. Preferably the hydrogen peroxide is produced enzymatically. Enzymatic production of hydrogen peroxide can be performed by using a number of different enzyme/substrate combinations, e.g. a combination of an oxidoreductase with an oxidizable substrate, for example;

10

glucose/glucose oxidase,
L amino acid/L amino acid oxidase,
galactose/galactose oxidase,
lactose/ β -galactosidase/glucose oxidase,
2-deoxyglucose/glucose oxidase.

15

It is possible to add both the substrate and/or the oxidoreductase to the system which is to be protected. It is also possible to use an enzyme which is already present in the substance for which protection is sought. For example, in milk the normally present xanthine oxidase can be used to generate hydrogen peroxide by addition of hypoxanthine as a substrate. This addition of substrate is necessary to activate the system.

25

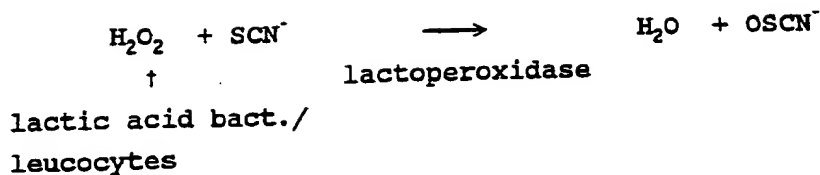
Combinations of different substrates and enzymes are equally effective and may give even better results. For example, the combination of glucose oxidase with β -galactosidase can be employed in lactose containing substances, β -galactosidase causes splitting of lactose, yielding galactose and glucose, the latter carbohydrate is then further oxidized by glucose oxidase.

30

The antimicrobial activity of this system is due to the formation of hypothiocyanate in the following reaction;

35

- 4 -



Raw milk contains all components which are essential for this reaction; thiocyanate and lactoperoxidase are present as such and hydrogen peroxide is produced by lactic acid bacteria or leucocytes. The thiocyanate is converted into hypothiocyanous acid (HOSCN) which at the pH of milk exists mainly in the form of the hypothiocyanate ion.

It may be useful in order to prolong the activity of the LP system to add hydrogen peroxide and/or if appropriate one of the other components of the system if they are limiting to the reaction. The addition of hydrogen peroxide in turn is limited by the effects which this molecule has on the activity of the lactoperoxidase and other proteins.

The hypothiocyanate ion reacts specifically with free sulfhydryl groups, thereby inactivating several vital metabolic enzymes and membrane proteins.

The hypothiocyanate has a bacteriostatic or bactericidal effect on a wide range of microorganisms. Activities of hypothiocyanate have been reported for example against, Pseudomonads, Enterobacteriaceae, Listeria, Yersinia, Campylobacter and Salmonella.

Milk preservation is an important application of this system. More generally, dairy products can be conserved using this system.

Other applications of the system in a more or less isolated form have been described. US patent 4,320,116 describes the use of this system in animal feedstuff and a method for treating bacterial infections in the gastrointestinal tract of mammals. Canadian patent application 1167381-A describes the use of this system in tooth-paste.

In general this system has the advantage of being food-grade; a wide spectrum of possible applications can therefore be envisioned.

5 One of the major problems with the use of the LP system is its short working time. The literature pertaining to this system thus far only reports activity ranging from a few hours to a maximum of a few days. The major factors responsible for this short working time are;

10 a) the uncontrolled (and high) production rate of hydrogen peroxide and,

b) the high reactivity of the hydrogen peroxide.

Due to its short working time, the LP system provides only temporary protection against microbial infection. The
15 protected substances are prone to renewed contamination and therefore the use of the LP system has been limited to date, to short term protection.

There is a need for a long term protection of food and feedstuffs by a naturally occurring antimicrobial system. The
20 present invention provides such a system.

Summary of the invention

25 The present invention discloses methods and means for increasing the working time of the lactoperoxidase system. To achieve this increased working time the invention discloses immobilized components of the lactoperoxidase system which provide for the sustained release of hydrogen peroxide.
30 Sustained release of hydrogen peroxide makes steady and continuous production of hypothiocyanate possible.

The present invention discloses immobilized components of the LP system. A system is disclosed which gives hydrogen peroxide production for at least 42 days.

35 The invention further discloses the application of the immobilized lactoperoxidas system components in food and feed.

Description of the figures

5 Figures 1-15 show the effect of the LP system on the following microorganisms: Escherichia coli ATCC 11229, Salmonella typhimurium ATCC 13311, Bacillus cereus IAM 1229, Staphylococcus aureus ATCC 6538 and Listeria monocytogenes RIVM 3 at the indicated pH values.

10 Figure 16 shows the effect of the lactoperoxidase system on Camembert cheese infected with Listeria.

 Figure 17 shows the production of d-gluconic acid in time from free glucose and from immobilized cornstarch.

15 Figure 18 shows the production of gluconic acid in time using immobilized cornstarch.

Detailed description of the invention

20 The present invention provides a composition for use in the sustained production of hydrogen peroxide comprising an oxidoreductase and or a corresponding substrate in an immobilized form.

 In its most general form, the present invention provides a method for obtaining sustained release of hydrogen peroxide by use of the composition. The hydrogen peroxide is slowly released and may be used for its antimicrobial activity as such. In order to exhibit antimicrobial activity, 30 the hydrogen peroxide must be present in relatively large amounts. Hypothiocyanate is a much more powerful antimicrobial agent than hydrogen peroxide. Antimicrobial activity has been reported for hydrogen peroxide at a concentration of 5mM, whereas 0.02mM hydrogen peroxide has 35 been reported to activate the LP system. The hydrogen peroxide is therefore preferably used to convert thiocyanate

into hypothiocyanate with the use of lactoperoxidase or another peroxidase.

Any thiocyanate salt may in principle be used. Commonly alkali metal salts such as sodium or potassium thiocyanate
5 are used.

To obtain slow release of hydrogen peroxide at least one of the components of the hydrogen peroxide generating system is immobilized. This can be the enzyme (the oxidoreductase) and/or the substrate (glucose, galactose or
10 other substrate) corresponding to the oxidoreductase used. It is also possible to immobilize more than one component.

The composition of the present invention may contain any oxidoreductase. Preferably the oxidoreductase is selected from the group consisting of
15 glucose oxidase, L amino acid oxidase, galactose oxidase, β -galactosidase/glucose oxidase, xanthine oxidase with a corresponding substrate. Combinations of oxidoreductases may advantageously be used in the present invention.

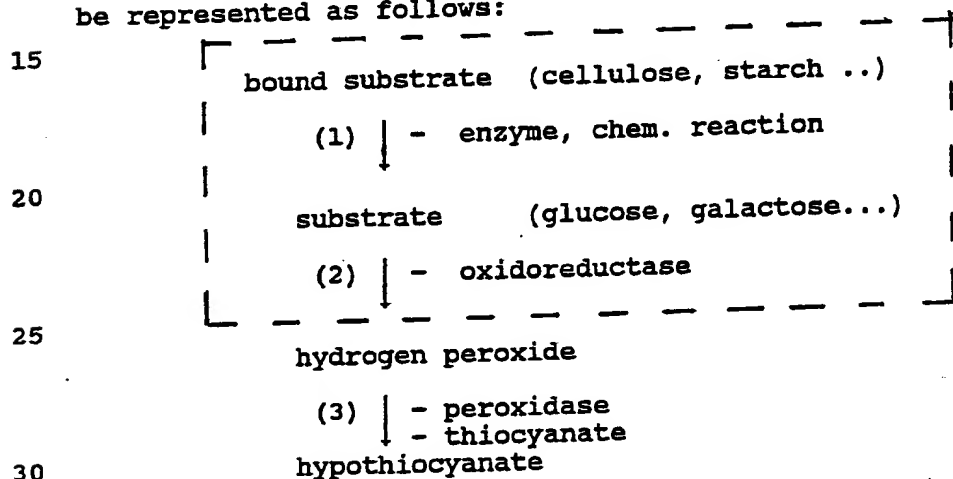
20 In the present invention the hydrogen peroxide is made available continuously and preferably at a steady-state level high enough to activate the lactoperoxidase system. To achieve this the substrate may for example be present in a slowly soluble form or it may be present in polymer form in
25 which case the substrate molecule is only available in usable form after an enzymatic or chemical reaction.

It is also possible to couple the above hydrogen peroxide production step with another reaction step in which the substrate is generated, thereby indirectly regulating the
30 hydrogen peroxide production rate by regulating the substrate release or production rate. An example of this is glucose which is obtained from cellulose by reaction with cellulase. Another example is the degradation of lactose using the combination of glucose oxidase with β -galactosidase. Yet
35 another example is the use of starch as a substrate necessitating the prior release of glucose. After release of the substrate the oxidoreductase reaction produces hydrogen

peroxide. It has been found that immobilization of the components provides a prolonged glucose release rate.

The hydrogen peroxide thus obtained is preferably used for increasing the effective working time of the lactoperoxidase system. We focus our discussion on the lactoperoxidase system since this system is the system of choice for food applications. However, it is recognized that other enzymes can equally well be employed according to the present invention to generate hydrogen peroxide, for example horseradish peroxidase and chloroperoxidase.

The system of the present invention can schematically be represented as follows:



Encircled is the part of the system wherein at least one of the components is immobilized.

To our knowledge it has not previously been attempted to use the LP system to obtain prolonged antimicrobial protection. Prolonged antimicrobial activity solves at the same time the problem of possible recontamination.

To date the LP system is generally used to treat the substance once, and subsequent reinfection is avoided by

physical separation of the 'protected' substance from sources of contamination.

The present invention makes outgrowth of reinfecting microorganisms during a longer period impossible.

5

In case the substrate used for the enzymatic reaction is also a substrate for one of the microorganisms present in the compositions to be protected, or in case the substrate is a substrate for other infecting microorganisms, it is preferable to add the substrate in a non-metabolisable form. Different options for using a non-metabolisable substrate present themselves;

- 1) the substrate can be immobilized, for example in the form of cellulose or starch. By producing and subsequently oxidizing glucose in situ, at a reaction rate that prevents accumulation, the growth of microorganisms can be prevented,
- 2) alternatively a non-metabolisable substrate as such can be employed; 2-deoxyglucose is an example.

20

The system of the present invention can be employed against a wide range of organisms. As indicated above the hypothiocyanate, which is produced in the lactoperoxidase reaction step, has been found to be active against a wide range of microorganisms including both gram-positive and gram-negative bacteria and fungi.

Activities of the hypothiocyanate have been reported for example against, Pseudomonads, Enterobacteriaceae, Listeria, Yersinia, Campylobacter, Salmonella, Streptococcus, Lactobacillus, Bacteroides, Flavobacterium and Fusobacterium.

30

The spectrum of activity of the present system can be increased by combining the system with other antimicrobial agents. Where apart from general protection, protection against a specific microorganism is required, it may be useful to add a bacteriocin to the system as described. This addition may be done either before or after immobilisation.

35

Suitable bacteriocins are known and include lantibiotics such as nisin.

The present invention exemplifies the use of the LP system against both gram-positive and gram-negative bacteria. Specifically it is shown that the system of the present invention is effective against the following microorganisms: Escherichia coli ATCC 11229, Salmonella typhimurium ATCC 13311, Bacillus cereus IAM 1229, Staphylococcus aureus ATCC 6538 and Listeria monocytogenes RIVM 3. Tests with these microorganism have been performed at different pH between 5 and 7. At all values the system works well, the preferred pH was 6.3.

The present invention focuses on step (2), the second step of the three-step process described above, that is the hydrogen peroxide production step. In order to obtain a steady-state level of hydrogen peroxide the amount produced should be kept constant. To achieve a constant hydrogen peroxide production level, the substrate for the peroxide formation reaction can be added in a controlled manner. Alternatively a limiting amount of enzyme, with an excess of substrate can be used.

The invention provides a method for controllably and slowly generating the hydrogen peroxide, this is achieved by the immobilisation of the enzymes or the substrates. Immobilisation methods are known. Suitable methods make use of for example Calcium alginate, gelatin or carrageenan. If necessary the immobilized material can be reinforced by cross-linking agents.

In the present invention some of the possible compositions are exemplified. Avicel[™] (cellulose) is immobilized together with cellulase and glucose oxidase in gelatin which is subsequently cross-linked with glutardialdehyde. In this system hydrogen peroxide is produced for at least 48 hours.

In another example cornstarch is immobilized together with α -amylase, amyloglucosidase and glucose oxidase in a combination of gelatin and alginate, with subsequent cross-linking with glutardialdehyde. It is shown that this system
5 is capable of releasing hydrogen peroxide for at least 42 days.

It is understood that the amounts of the components and the composition itself in the system will vary depending on the specific application. The coupling of the exemplified
10 systems with lactoperoxidase/thiocyanate will increase the effectivity of the antimicrobial composition.

In liquid form minimal amounts of the LP components are as follows;

glucose oxidase (Gist-brocades) 0.8 mg/l,
15 lactoperoxidase (Biopole) 1 mg/l,
hydrogen peroxide 0.02 mM,
SCN⁻ 0.02mM.

In general the molar ratio between peroxide and
20 thiocyanate is smaller than 4 and preferably it is 1-2.

The lactoperoxidase is present in amounts varying from 1 - 200mg/l. Activities of the enzymes are as follows;

glucose oxidase, 36.000 units/g (pH=6, T=14°C), wherein 1U =
1 μ mol hydrogen peroxide /min,
25 lactoperoxidase 481.000 ABTS units/g (pH=6, T=25°C)
(ABTS method, Childs et al. Biochem.J. (1975) 145 93-103).

Finally the present invention discloses a food product
30 which when treated with 10²-10⁵ microbial cells per g between 2-10 days after preparation and which is subsequently kept at normal growth conditions for the infecting microorganism does not give rise to outgrowth of this microorganism and wherein protection is due to sustained hydrogen peroxide production.
35 Specifically, it is also shown that the LP system is effective against Listeria, when applied on cheese (Camembert).

Specific amounts of cells and growth conditions may of course vary depending on the nature of product and the microorganism which is employed.

5 When practising the invention, the substance to be protected is mixed with quantities of the reagents in such a way that the hydrogen peroxide will be generated in such an amount per unit time that a steady-state concentration is achieved.

10

The system as described, provides its antimicrobial protection for at least 10 days, preferably at least 20 days and more preferably up to 50 days.

15 Utility of the invention.

The system can be applied to food and feed conservation. With respect to this application, it can be used in a liquid, for example in (cheese-)milk, but it will be equally effective when applied to the surface of for
20 example cheese. The system can also be applied as a longterm cleaning agent in specific applications. It is understood that the amounts of the components in the system will vary depending on the specific application.

The use of this system can be envisioned in the
25 decontamination of carcasses (bovine, fish, shrimps), surface treatment of food (cheese, butter), treatment of fresh vegetables, cosmetics, wound treatment, toothpaste, decontamination of machines (icemachines, milkshake machines) or more broadly equipment used in food processing in plants
30 or in area wherein food is prepared in large amounts (hospitals, restaurants and the like), decontamination of udders, silage and in feedstuff.

Experimental

Hydrogen peroxide analysis

5 Measurement of the amount of hydrogen peroxide was performed by a modification of the method described Mottola et al. Anal. Chem. 42:410-411 (1970).

Briefly, in a 1 cm cuvet the following solutions were mixed;

- 10 50 μ l sample containing hydrogen peroxide (0.2-1 mM)
200 μ l leuco-crystal-violet (LCV) solution (0.5-1 mg/ml in 0.5% HCl)
1.6 ml Sodium acetate buffer (0.5 mM pH 4.5)
100 μ l lactoperoxidase (2 mg/ml) or HRP
15 (Horse Radish Peroxidase)

In the presence of thiocyanate, lactoperoxidase can not be used to obtain accurate measurements in this assay. However, under these conditions horse radish peroxidase works
20 well.

Color development was followed at 596nm.

Composition of media

25 Minimal medium contained the following substances per liter;

K_2HPO_4 , 14g; KH_2PO_4 , 6g; $(NH_4)SO_4$, 2g; Trisodiumcitrate. $2H_2O$, 1g; $MgSO_4 \cdot 7H_2O$, 0.2mg; $MnSO_4 \cdot 2H_2O$, 5g; L-glutamic acid, 2g; NaOH, 0.8g; 50ml 10% Casamino acid solution (Difco), 20ml 50%
30 glucose solution and 10ml Vitamin solution.

Vitamin solution contained per liter;

2mg biotin; 2mg folic acid; 10mg pyridoxine HCl B6; 5mg thiamine HCl B1; 5mg riboflavin B2, 5mg nicotinic acid; 0.1mg
35 vitamin B12; 5mg p-aminobenzoic acid; 5mg DL Calciumpenthotenate.

Cheese milk medium (CM medium) contained per liter;
15g Caseinehydrolysate, 3g tri-sodiumcitrate, 3g lactose,
5 3.5g lactate, 5g tryptose and 50mM phosphate buffer (pH 5, 6
or 7).
After sterilisation glucose was added.

10

Examples

Example 1

15 Activity of the lactoperoxidase system against specific microorganisms

The activity of the lactoperoxidase system against five
different microorganisms was tested using glucose oxidase/
20 glucose to generate hydrogen peroxide.

The microorganisms used were the following:

Gram-negative: Escherichia coli ATCC 11229
Salmonella typhimurium ATCC 13311
Gram-positive: Bacillus cereus IAM 1229
25 Staphylococcus aureus ATCC 6538
Listeria monocytogenes RIVM 3

E.coli, S.tyhimurium, B.cereus and S.aureus were
incubated at the desired pH in minimal medium.
30 L.monocytogenes was incubated in cheese milk medium.
After overnight culture the cells were used to inoculate the
main culture to a density of 10^3 - 10^5 cells/ml. The incubation
temperature was 37°C. The pH was 5.2, 6.3 or 7.2 (for
L.monocytogenes; 5.0, 6.0 and 7.0). To these cultures the
35 given substances were added to the indicated final
concentrations:

- 15 -

- SCN⁻ 100mg/l (sodium salt, Merck);
- lactoperoxidase 20mg/l (Biopole);
- glucose-oxidase 1.5 mg/l (Gist-brocades);
- glucose 10 g/l (BDH).

5 The control cultures contained the same substances without glucose-oxidase.

The number of viable cells was followed in time and determined by plating several dilutions on BHI plates.

10

The hydrogen peroxide concentration was monitored during these experiments using the method outlined in Experimental. It could be concluded that using the concentrations mentioned above the hydrogen peroxide was
15 never present in an amount sufficient to have any microbial effect as such. Thus, the antimicrobial effects described could completely be attributed to the hypothiocyanate.

Results are shown in Figures 1-15.

20 E.coli (Fig. 1-3)

- pH=7.2 - cells killed between 8 and 24 hours
- control continues to grow after a lag phase of 4 hours
- pH=6.3 - cells killed after 4 hours
- 25 - control continues to grow after 4 hours
- pH=5.2 - cells killed after 2 hours
- control continues to grow after 6 hours

S.typhimurium (Fig. 4-6)

- 30 pH=7.2 - cells killed between 6 and 24 hours
- control continues to grow after 4 hours
- pH=6.3 - cells killed within 2 hours
- control continues to grow after 6 hours
- pH=5.2 - cells killed after 4 hours
- 35 - control continues to grow after 4 hours

- 16 -

S.aureus (Fig. 7-9)

- 5 pH=7.2 - cells not completely killed
 - control continues to grow after 8-24 hours
 pH=6.3 - cells killed within 4 hours
 - control as with pH=7.2
 pH=5.2 - cells killed within 6 hours
 - control as with pH=7.2

B.cereus (Fig. 10-12)

- 10 pH=7.2 - cells killed within 2 hours
 - control continues to grow after 2 hours
 pH=6.3 - cells killed as with pH=7.2
 - control continues to grow after 4 hours
 pH=5.2 - cells killed between 6 and 24 hours
15 - control continues to grow after 6 hours

L. monocytogenes (Fig. 13-15)

- pH=7.0 - cells killed between 5 and 24
 hours
20 - control continues to grow after 5 hours
 pH=6.0 - cells killed between 3 and 24 hours
 - control continues to grow after 2 hours
 pH=5.0 - cells killed between 3 and 24 hours
 - control does not grow at this pH.

25

It can be concluded that all tested microorganisms are adequately killed under the given experimental conditions, except for S.aureus at pH=7.2.
Overall pH=6.3 is the optimal pH.

30

Example 2Sustained release of hydrogen peroxide

35

5g of Avicel^(tm) (a crystalline polymer consisting of cellulose, Serva) was suspended in 45 ml of an aqueous

- 17 -

solution of gelatin, at 30°C. Subsequently 20mg cellulase (Gist-brocades, Maxazym[™] CL 2000) and 25mg glucose oxidase (Gist-brocades) were added. This gelatin-polymer-enzyme suspension was added to 100ml of a stirred cornoil (Brocacef) solution at 30°C. The water in oil suspension was cooled to 10°C. The particles are cross-linked by slowly adding (in 60 min) 1.03 g glutardialdehyde (Merck) in 8.25 ml water.

0.05g Tween 80 in 5 ml water was added and stirring was continued for 5 min.

Subsequently the particles were separated from the oil phase by addition of 1000ml water and the particles were washed twice with the same amount of water. The particles were stable and insoluble in water.

The release of hydrogen peroxide was followed in time using the method described in the Experimental section. The experiment was performed by incubating 5 g of the particles in 50ml buffer (pH=5.0) in a stirred reactor vessel at room temperature.

The results are shown in Table 1. It can be concluded that hydrogen peroxide production is sustained and constant.

Table 1. Generation of hydrogen peroxide

time (h)	$\mu\text{mol H}_2\text{O}_2$ /h/g particles	
24	0.020	0.021
48	0.022	0.019

Example 3

Effect of the lactoperoxidase system on Camembert cheese infected with Listeria monocytogenes

Camembert cheese frozen 1 day after production was kept frozen at -50°C. Freezing and thawing had no visible effect

on the cheese flora. Cheeses were put in refrigerator boxes with a volume of 1 liter and the relative humidity was kept at 95% using a glycerol/water mixture.

The boxes were incubated at 14°C. After 5 days cheeses
5 were treated with Listeria monocytogenes DSM 20600 at 100 cells per gram cheese (in 0.5ml). After 4 hours the cheeses were treated at one side with 0.6ml of LPS solution (100mM glucose (BDH), 20mM NaSCN (Merck), 200mg/l lactoperoxidase (Biopole) and 50mg/l glucose oxidase (Gist-brocades)).
10 Control cheeses were treated with milli-Q water.

The number of Listeria were counted at t=0 and after 1, 2 and 5 days in duplo. Counting was performed by diluting 17 g of cheese two times in 2% tri-sodiumcitrate. After
15 homogenisation in a Stomacher the suspension was diluted in a physiological salt solution.

0.1 ml of different dilutions were brought onto Palcam plates (Merck). Plates were grown at 30°C during one day and colonies were counted.

20 The result is shown in Figure 16 and it can be concluded that the LPS system works well under application conditions.

25

Example 4

Use of immobilized cornstarch as a glucose source I

A suspension consisting of 10% (w/w) cornstarch in 8%
30 (w/w) gelatin and 1% (w/w) alginate was prepared. The mixture was kept at 30°C and 0.05% α -amylase (Gist-brocades, Maxamyl™, 6300 TAU/g), 0.05 % amyloglucosidase (Gist-brocades, Amigase™ TS, 25000 AGI/ml) and 0.05% glucose oxidase (Gist-brocades) (all on w/w basis) were added.

35 Subsequently the suspension was poured into two volumes of cornoil containing 1% (w/w) Span 80. The mixture was heavily stirred using a turbin rotor. After 5 minutes the

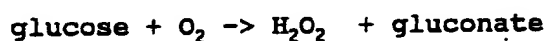
- 19 -

temperature was lowered to 15°C and after coagulation 8.25 ml cross-link mixture was added per 50 g of formulation. Cross-link mixture consisted of 88.5% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in ethanol (40 g per 100ml ethanol) and 11.5% glutardialdehyde (25% w/w).

After 60 minutes the immobilisation product-oil emulsion was stirred in an excess water for 5 minutes and the oil was decanted. The immobilisation product was subsequently washed twice with an excess of water and finally isolated by fractionated sieving.

In order to follow the hydrogen peroxide production rate production of gluconic acid was measured. Gluconate is a product of the hydrogen peroxide forming reaction:

15



Two open, stirred 100ml reaction vessels were used to follow the D-gluconic acid production at room temperature. Vessel one contained 2.5 g of immobilisation product in 25 ml 0.1 M sodium acetate buffer, pH 5.

Vessel two contained 0.277 g glucose and 1.38 g glucose oxidase in 25 ml 0.1 M sodium acetate buffer, pH 5.

D-gluconic acid was measured using an enzymatic test kit from Boehringer Mannheim (cat. no. 428.191).

The results are presented in Figure 17 it can be seen that without immobilisation and use of free glucose the D-gluconic acid production stops after about 50 hours whereas D-gluconic acid production and hence hydrogen peroxide production continues for more than 200 hours when immobilized starch is used as glucose source.

The maximum amount of gluconic acid which could be obtained from the amount of starch used in this experiment was 60 mM.

Example 5Use of immobilized cornstarch as a glucose source II

5 50 g of cornstarch was suspended in 200 ml water and heated to 85°C. The slurry was kept at this temperature for 15 minutes with continues stirring. A solution of 50 g gelatine in 200 ml was added. After cooling the suspension to about 40°C, 125 mg amyloglucosidase (Amigase™ TS), 250 mg
10 glucose oxidase and 1250 mg lactoperoxidase were added. Thereafter 6 ml glutardialdehyde (25% w/w) was added with continuous stirring. The gel was homogenized with a blender. After homogenisation 2 l, 0.2 M sodium acetate containing 0.7% glutardialdehyde was added. The mixture was stirred for
15 15 minutes at 15°C. The product was sieved and washed twice with a tenfold volume of water. The product was subsequently dried in a fluid bed dryer at 39°C to 94% (w/w) dry weight. Finally the dried particles were milled in a high speed hammer mill to a particle size of approximately 20 microns.

20

150 mg of the dried particles (20 microns) was suspended in 149 ml 0.1 M sodiumphosphate buffer pH=7.5, containing 4.5% (w/v) NaCl and 1.0 ml 400 mM NaSCN.

Incubation was in a shaking waterbath at 7°C with such
25 a speed that the particles were kept in constant motion and that aeration was assured. Gluconic acid production was followed in time using the previously mentioned Boehringer test kit.

Figure 18 shows the results. Under the given conditions
30 hydrogen peroxide can be generated for at least 42 days at a constant rate.

The maximum amount of gluconic acid which could be obtained from the amount of starch used in this experiment was 3 mM.

35

CLAIMS

- 5 1. A method for obtaining sustained release of hydrogen peroxide through the reaction of at least one oxidoreductase with its corresponding substrate characterized in that the oxidoreductase and/or the substrate is immobilized.
- 10 2. A method according to claim 1, characterized in that the immobilization is performed with gelatin, alginate, carrageenan.
3. A method according to claim 1 or 2 wherein the
15 oxidoreductase is selected from the group consisting of glucose oxidase, L amino acid oxidase, galactose oxidase, β -galactosidase/glucose oxidase and xanthine oxidase.
4. A method according to any one of the previous claims
20 wherein a combination of at least two oxidoreductases is employed.
5. The method of any one of the previous claims wherein the hydrogen peroxide is used to convert thiocyanate into
25 hypothiocyanate in the presence of a peroxidase.
6. The method of claim 5 wherein the peroxidase is lactoperoxidase.
- 30 7. Use of the method of any one of the previous claims as an antimicrobial system in the preservation of food or feed.
8. Use of the method of claim 7 in the conservation of
35 cheese.

9. Use of the method of claim 7 against gram-positiv or gram-negative bacteria or fungi.

10. Use of the method of claim 9 against the following
5 microorganisms; Escherichia coli, Salmonella typhimurium,
Bacillus cereus, Staphylococcus aureus or Listeria
monocytogenes.

11. Use of an oxidoreductase in a reaction leading to
10 sustained release of hydrogen peroxide.

12. Use according to claim 11 wherein the
oxidoreductase is selected from the group consisting of
glucose oxidase, L amino acid oxidase, galactose oxidase and
15 β -galactosidase/glucose oxidase and xanthine oxidase.

13. A composition containing an oxidoreductase and/or a
substrate in an immobilized form.

20 14. A composition according to claim 13 characterized
in that the oxidoreductase is selected from the group
consisting of glucose oxidase, L amino acid oxidase,
galactose oxidase and β -galactosidase/glucose oxidase and
xanthine oxidase.

25 15. A composition containing starch, amyloglucosidase,
 α -amylase and an oxidoreductase in an immobilized form.

30 16. A composition according to claim 15, wherein the
components are immobilized in alginate, gelatine or
carrageenan.

17. A food product containing a composition according
to any one of the claims 13 to 16.

35 18. A cheese containing a composition according to any
one of the claims 13 to 16.

Effect LPS on E.coli ATCC 11229 Minimal medium pH 7.3, 37°C

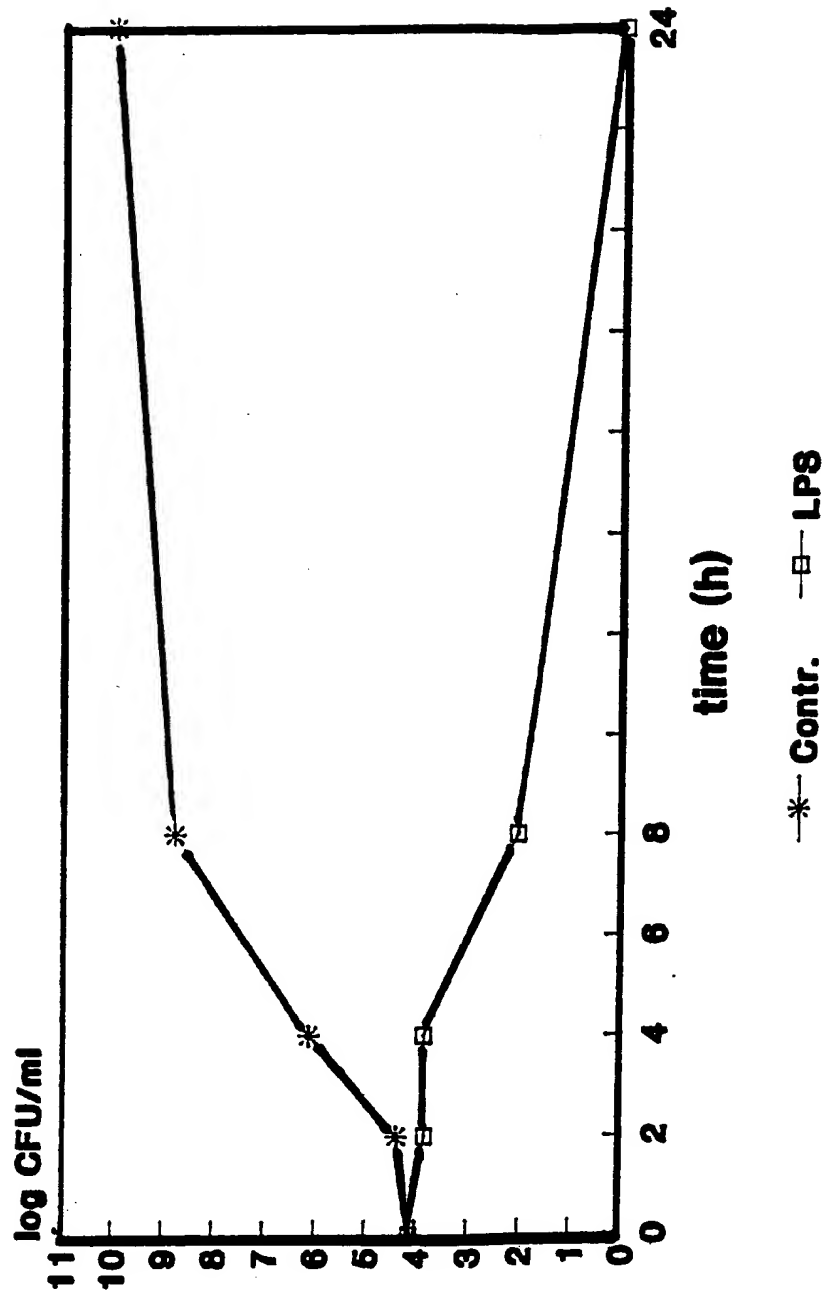
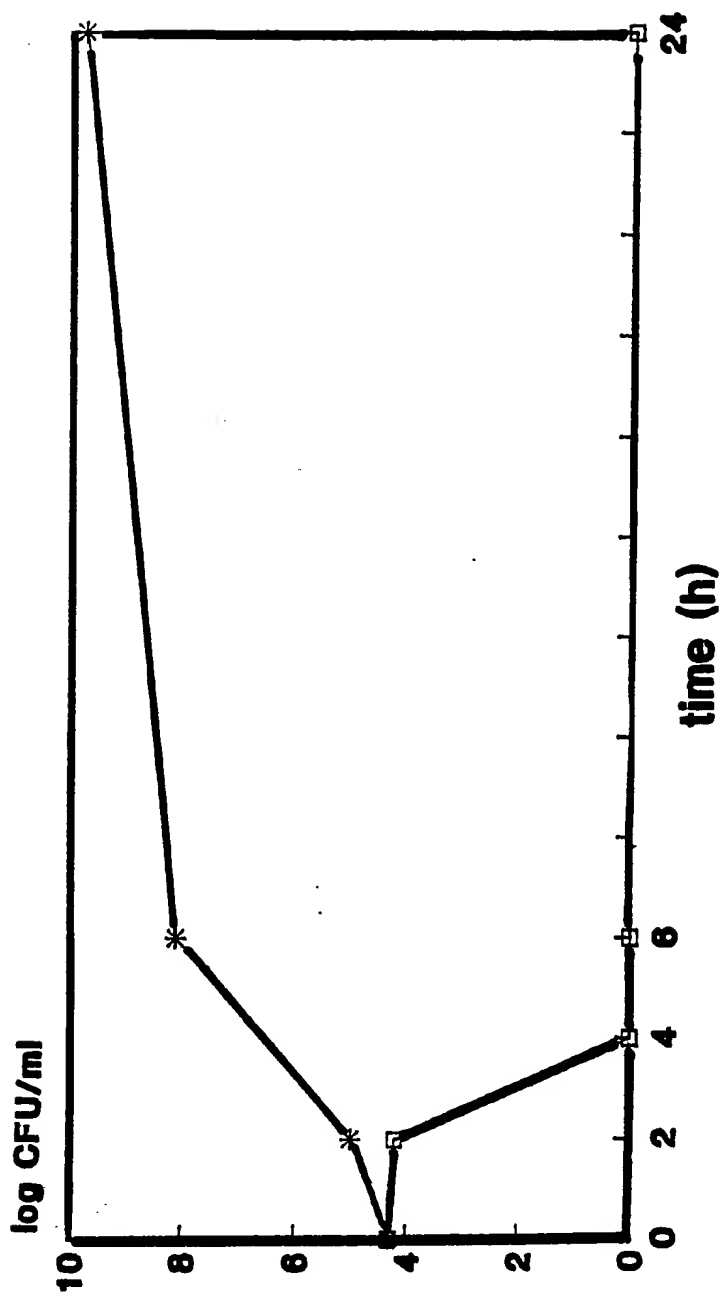


Figure 1

Effect LPS on E.coli ATCC 11229
Minimal medium pH 6.3, 37°C



—*— Contr. —□— LPS

Figure 2

Effect LPS on E.coli ATCC 11229
Minimal medium pH 5.3, 37°C

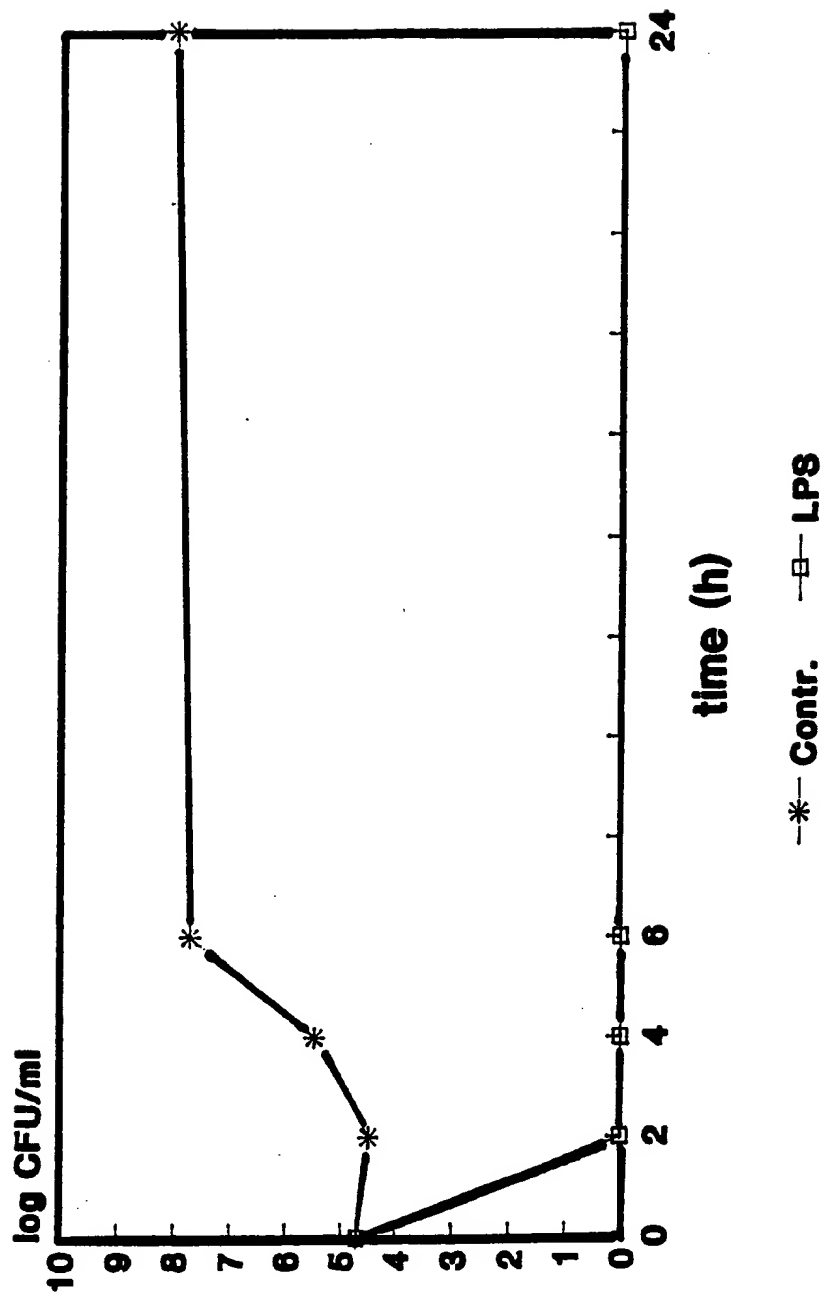


Figure 3

Effect LPS on Salm.typhimur. ATCC 13311
Minimal medium PH 7.2, 37°C

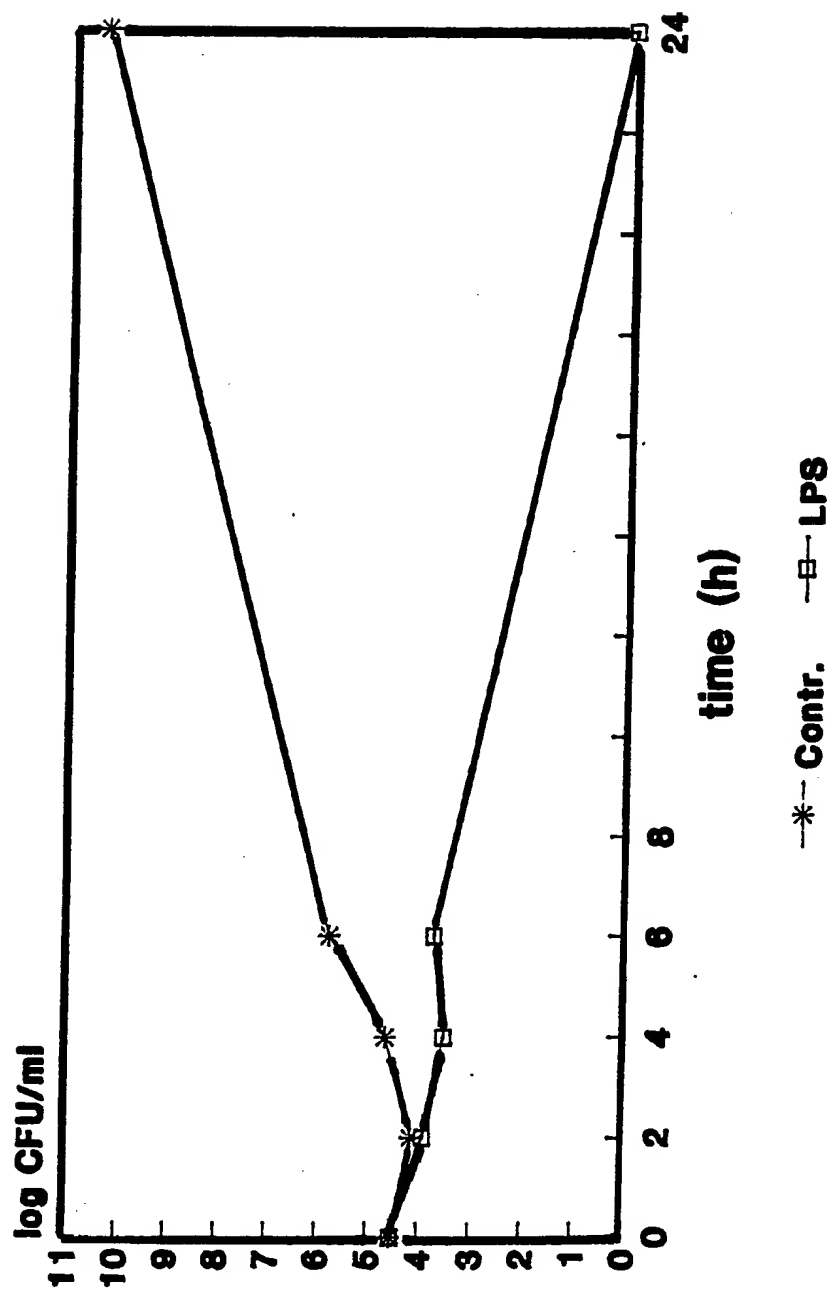


Figure 4

Effect LPS on Salm.typhimur. ATCC 13311
Minimal medium PH 6,37°C

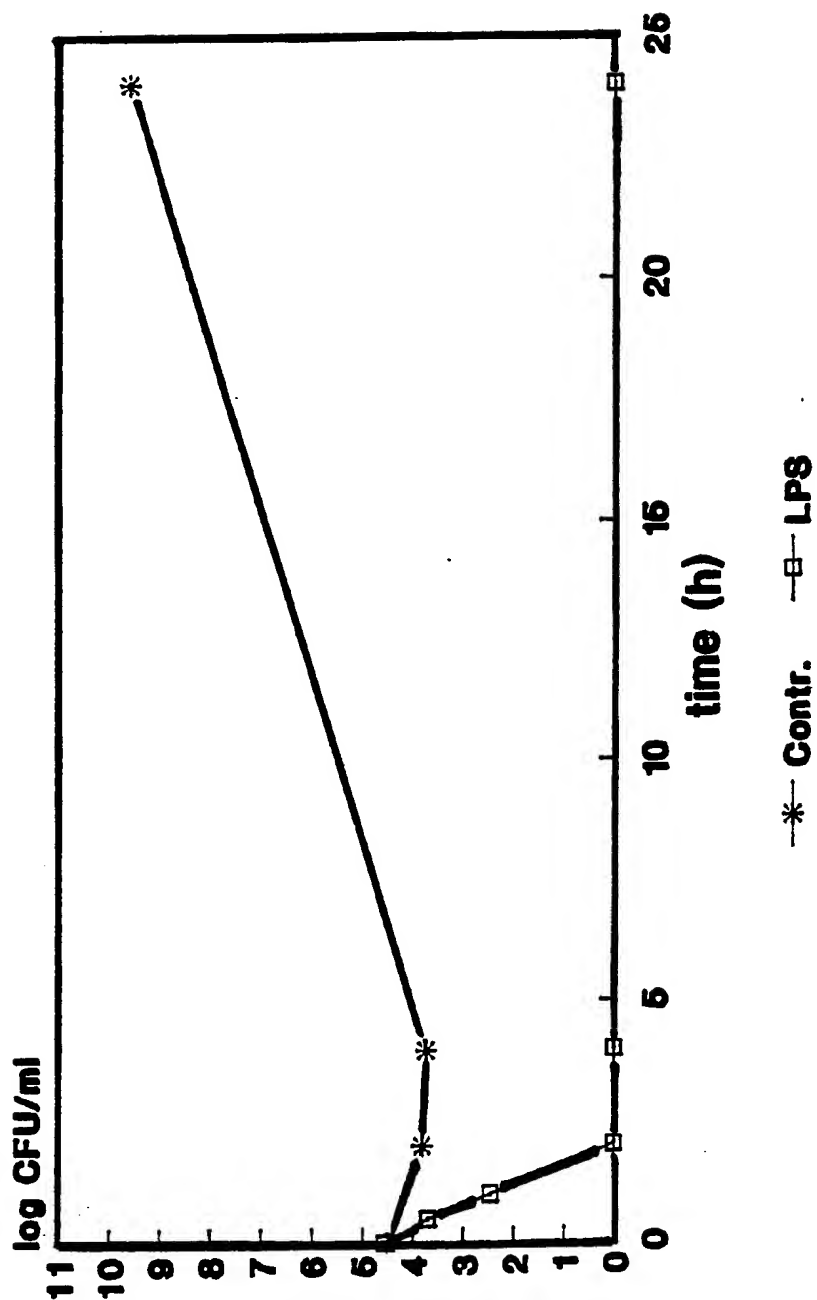


Figure 5

6/18

Effect LPS on Salm.typh. ATCC 13311
Minimal medium pH 5.2, 37°C

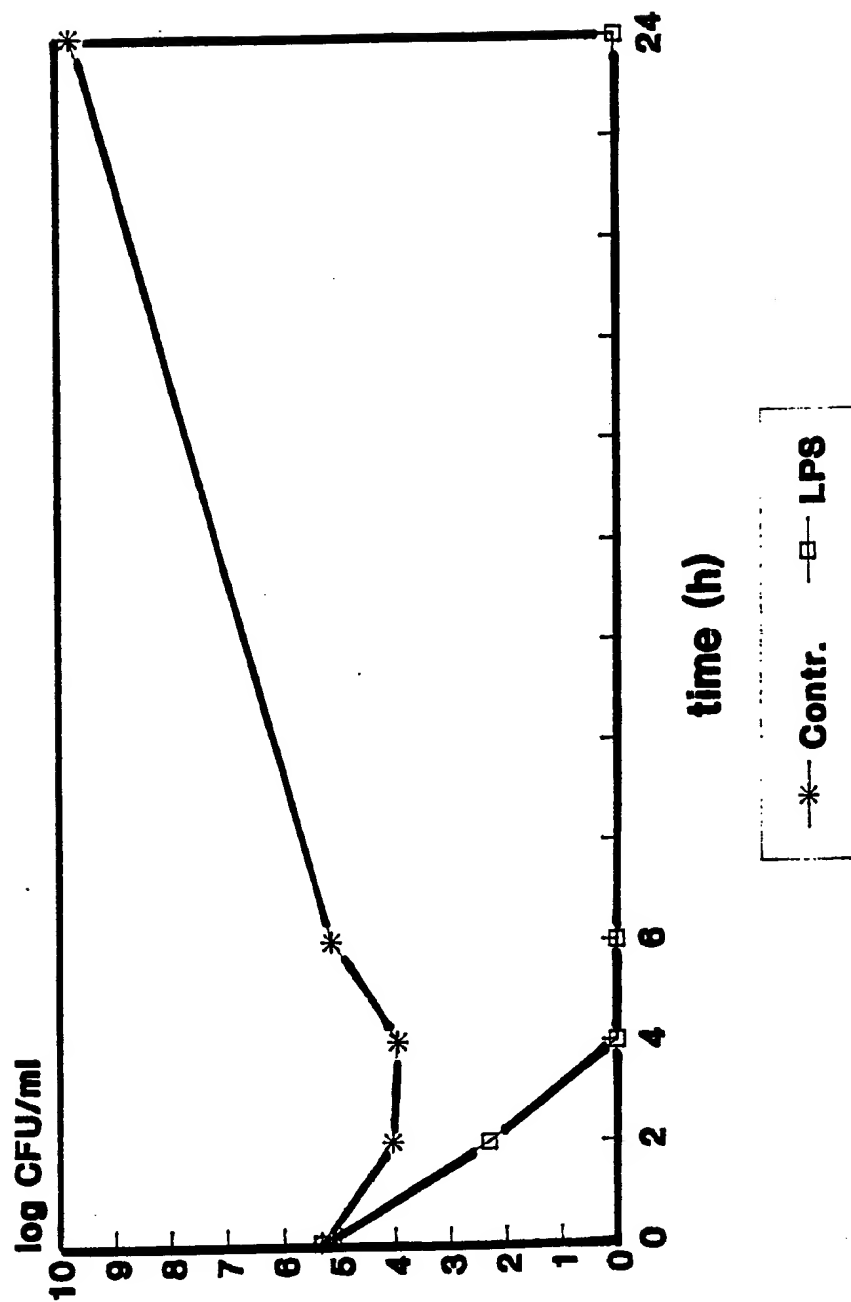


Figure 6

Effect LPS on Staph.aureus ATCC 6538

Minimal medium pH 7.2, 37°C

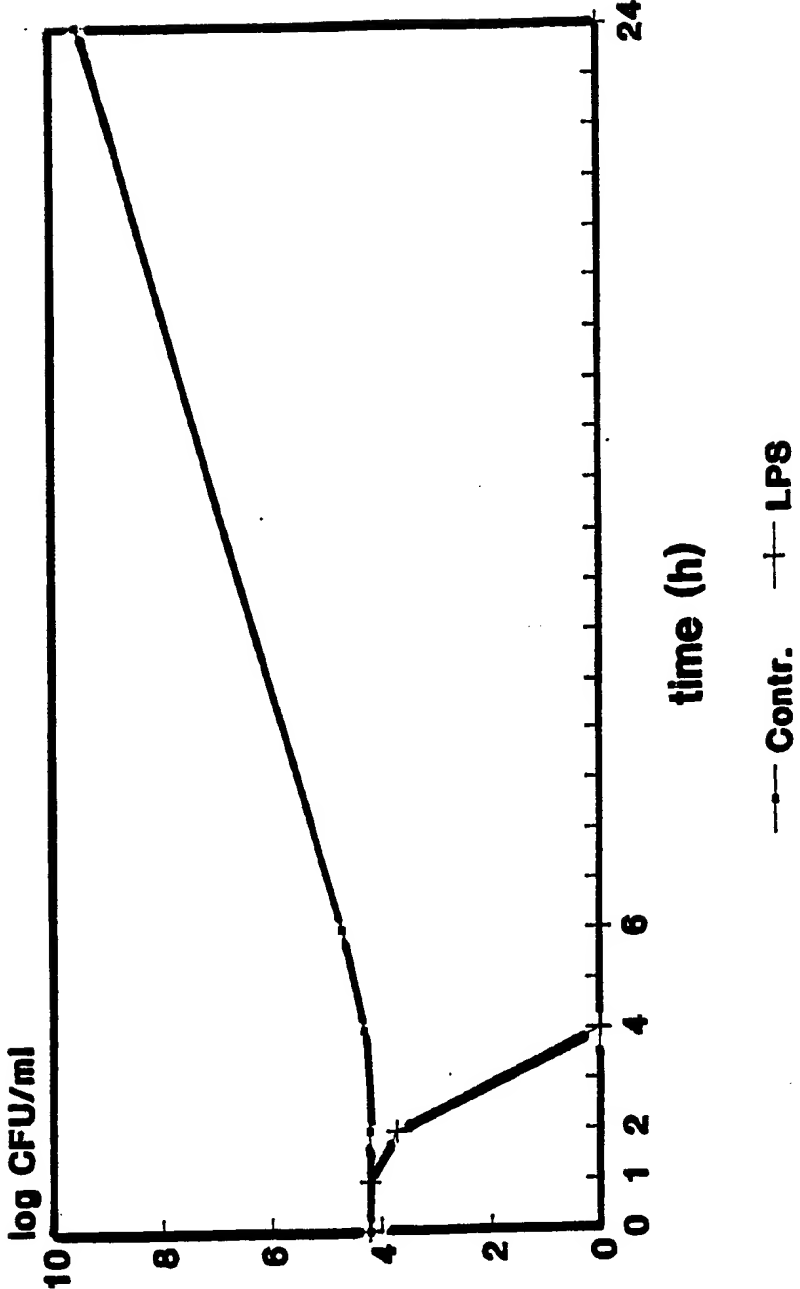


Figure 7

Effect LPS on Staph.aureus ATCC 6538
Minimal medium pH 6.3, 37°C

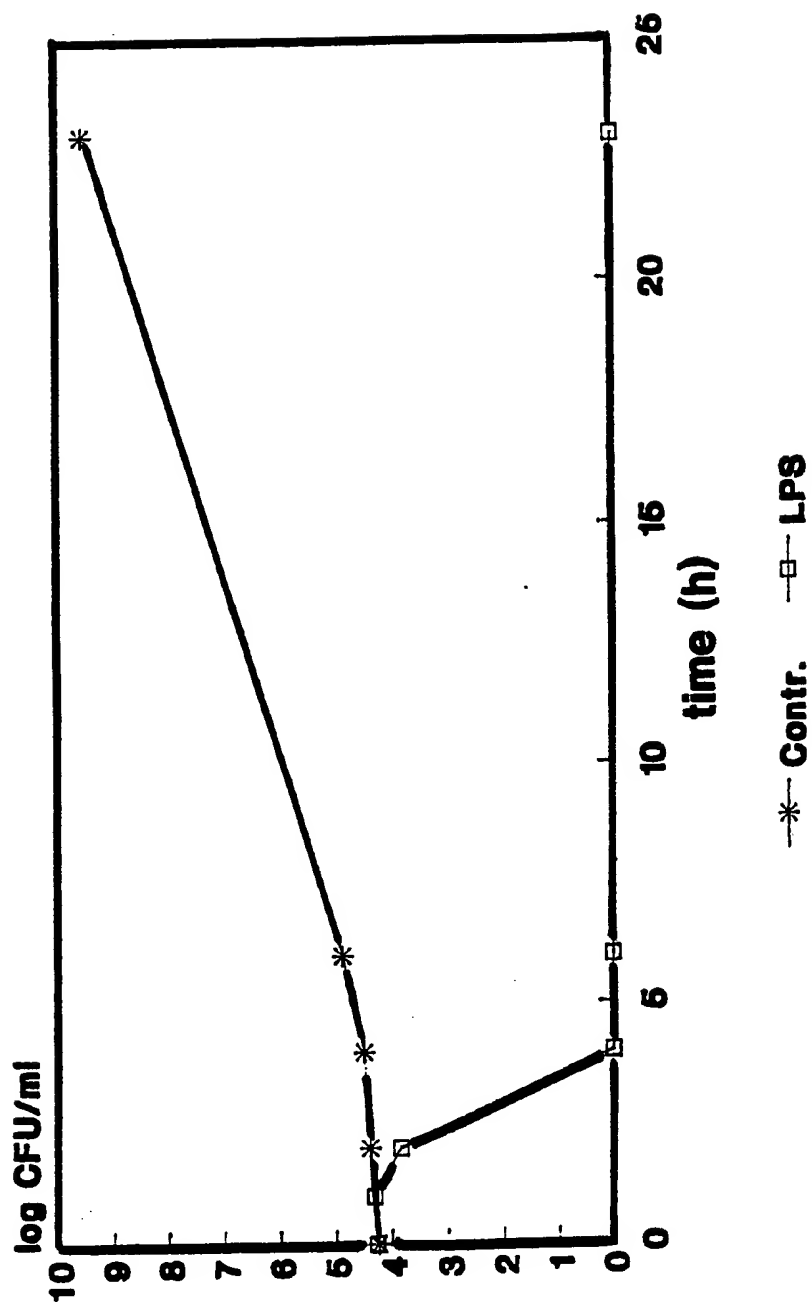


Figure 8

9/18

Effect LPS on Staph.aureus ATCC 6538
Minimal medium pH 5.2, 37°C

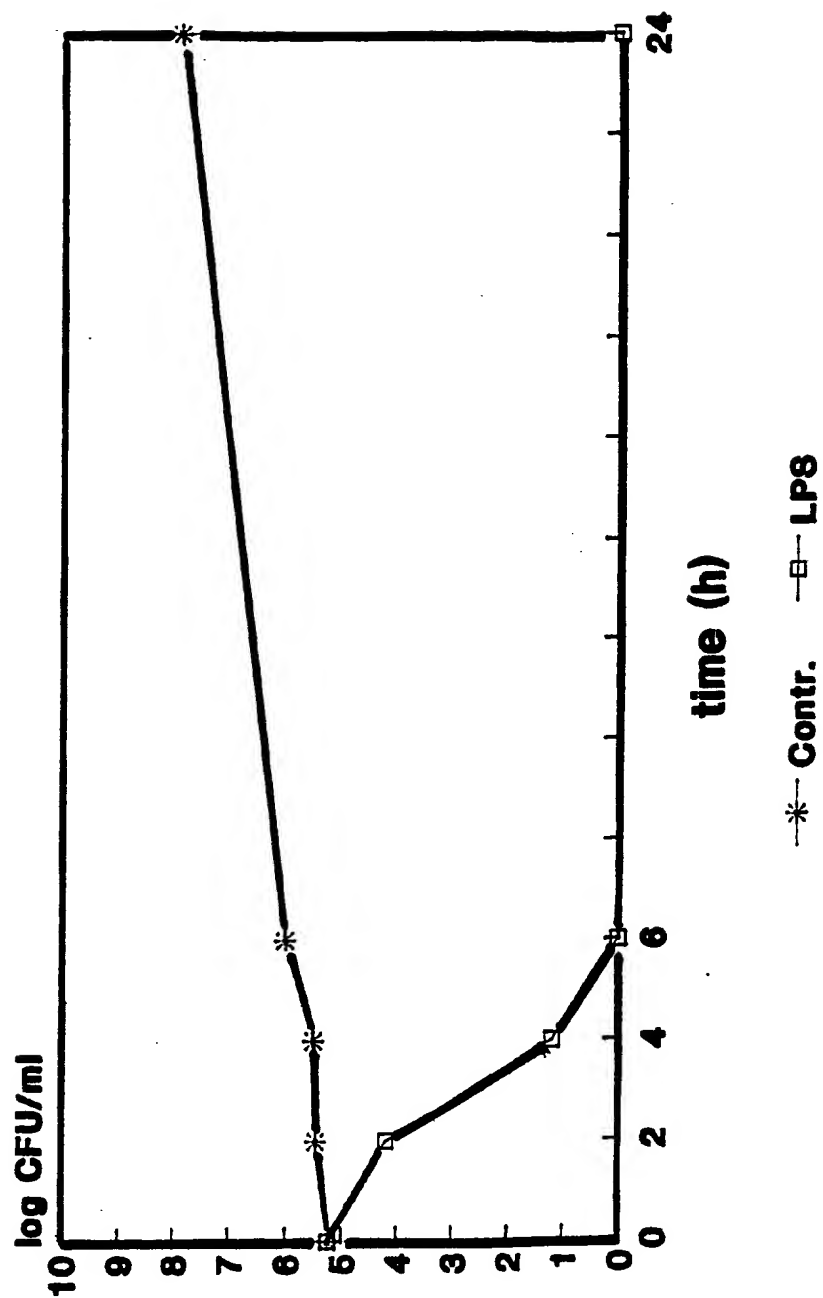


Figure 9

10/18

Effect LPS on Bac.cereus IAM 1229
Minimal medium pH 7.3, 37°C

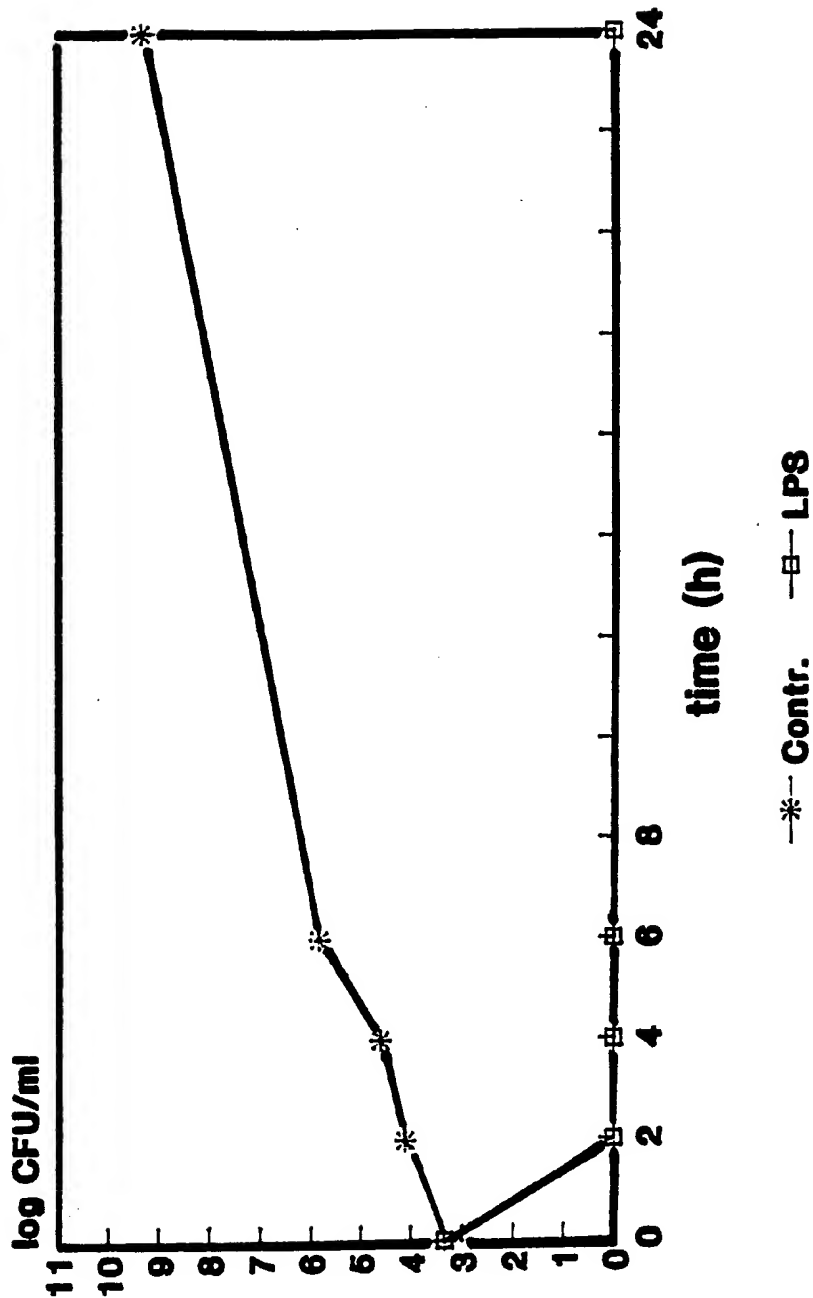


Figure 10

11/18

Effect LPS on Bac.cereus IAM 1229

Minimal medium pH 6.3, 37 C

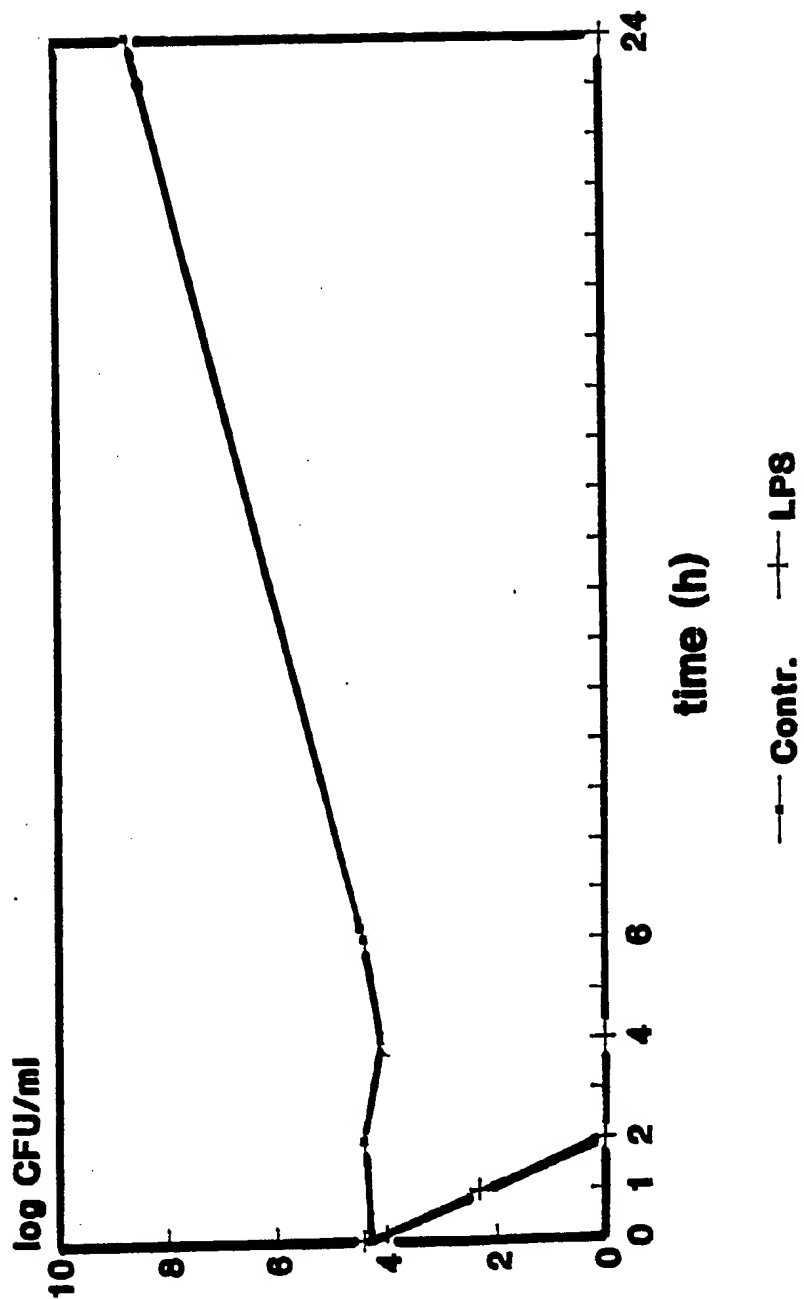


Figure 11

12/18

Effect LPS on *Bac.cereus* IAM 1229
Minimal medium pH 5.2, 37°C

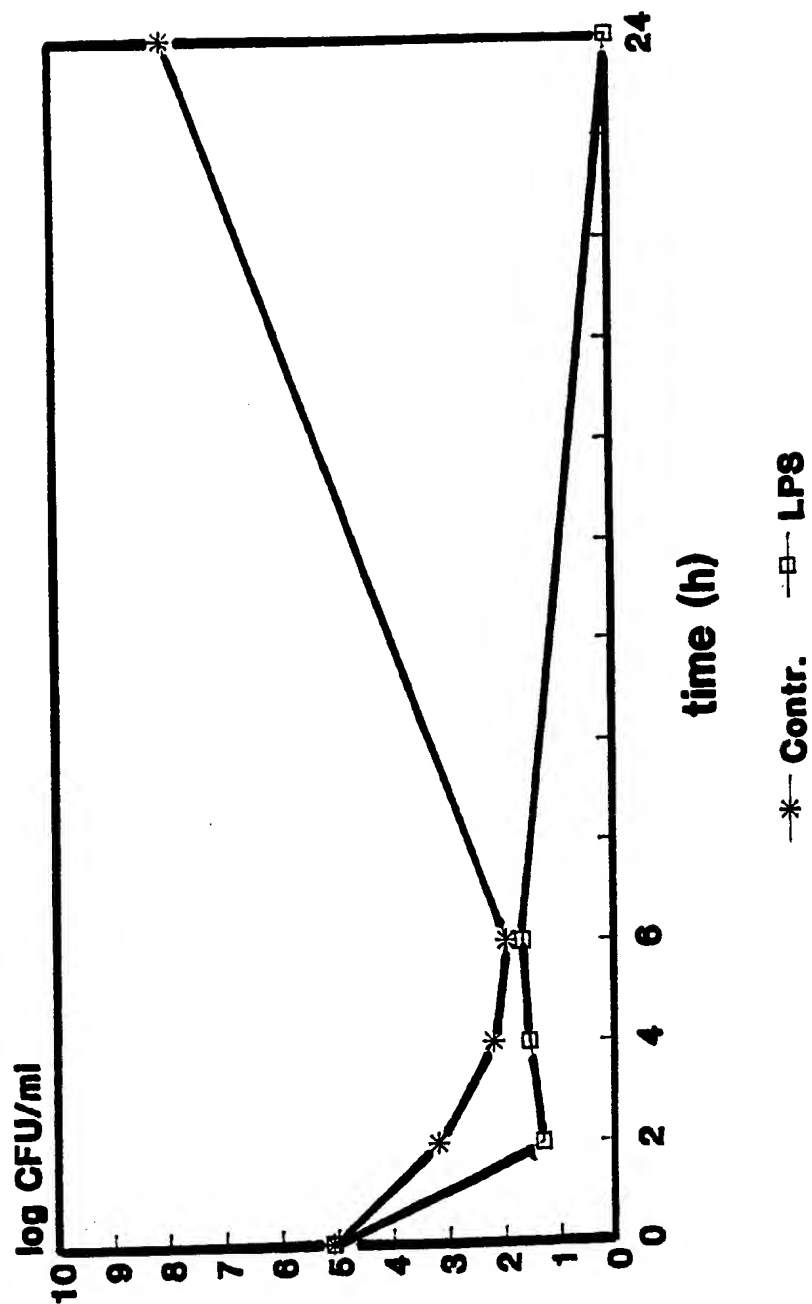


Figure 12

Effect LPS on *List.monocytogenes* RIVM 3

CM medium pH 7, 37°C

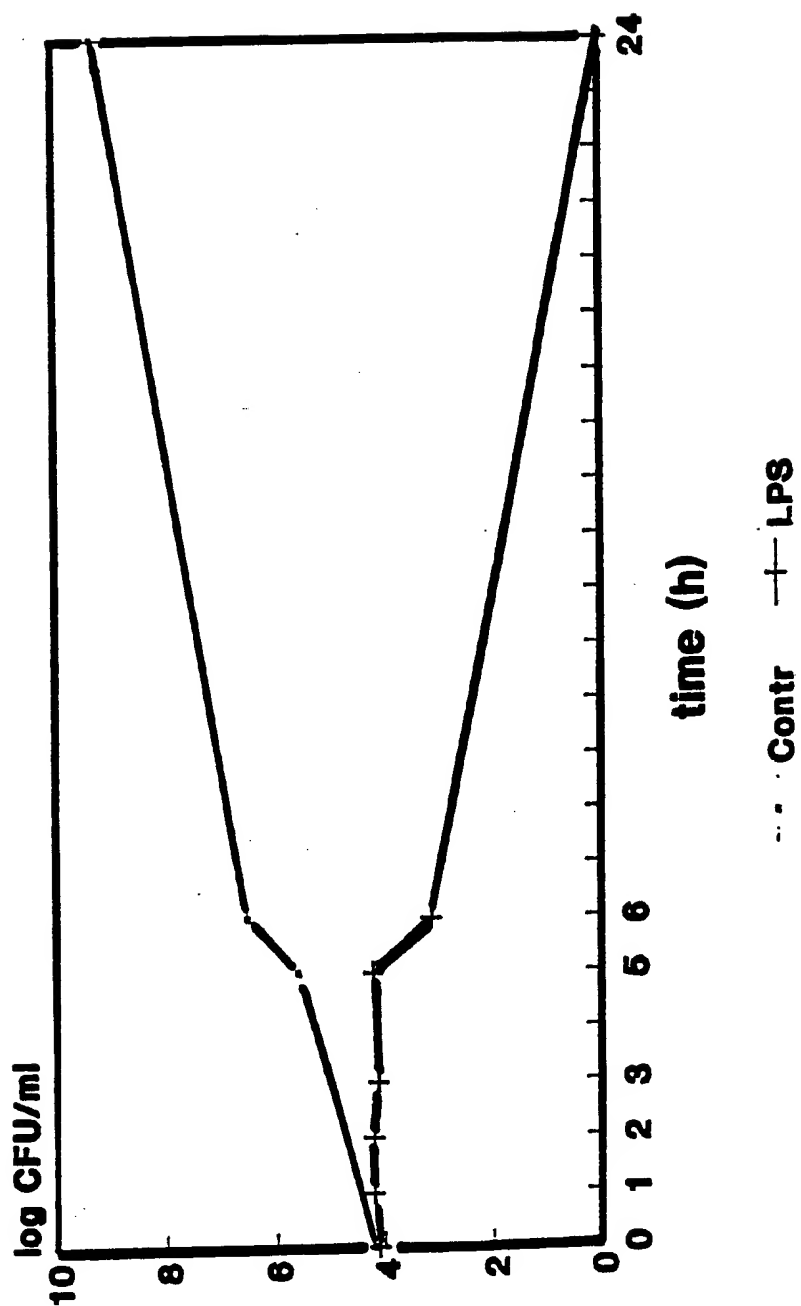


Figure 13

Effect LPS on List.monocytogenes RIVM 3

CM medium pH 6, 37°C

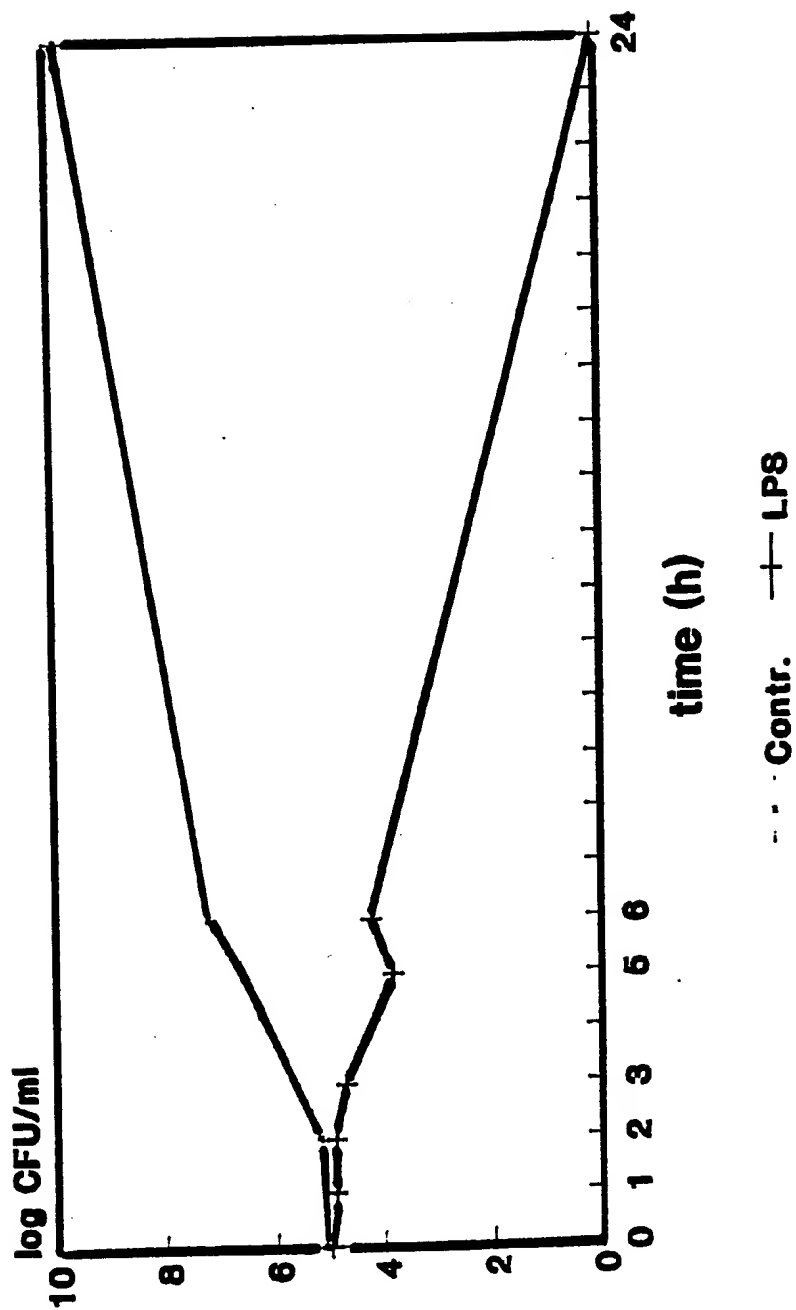


Figure 14

Effect LPS on *List. monocytogenes* RIVM 3

CM medium pH 5, 37°C

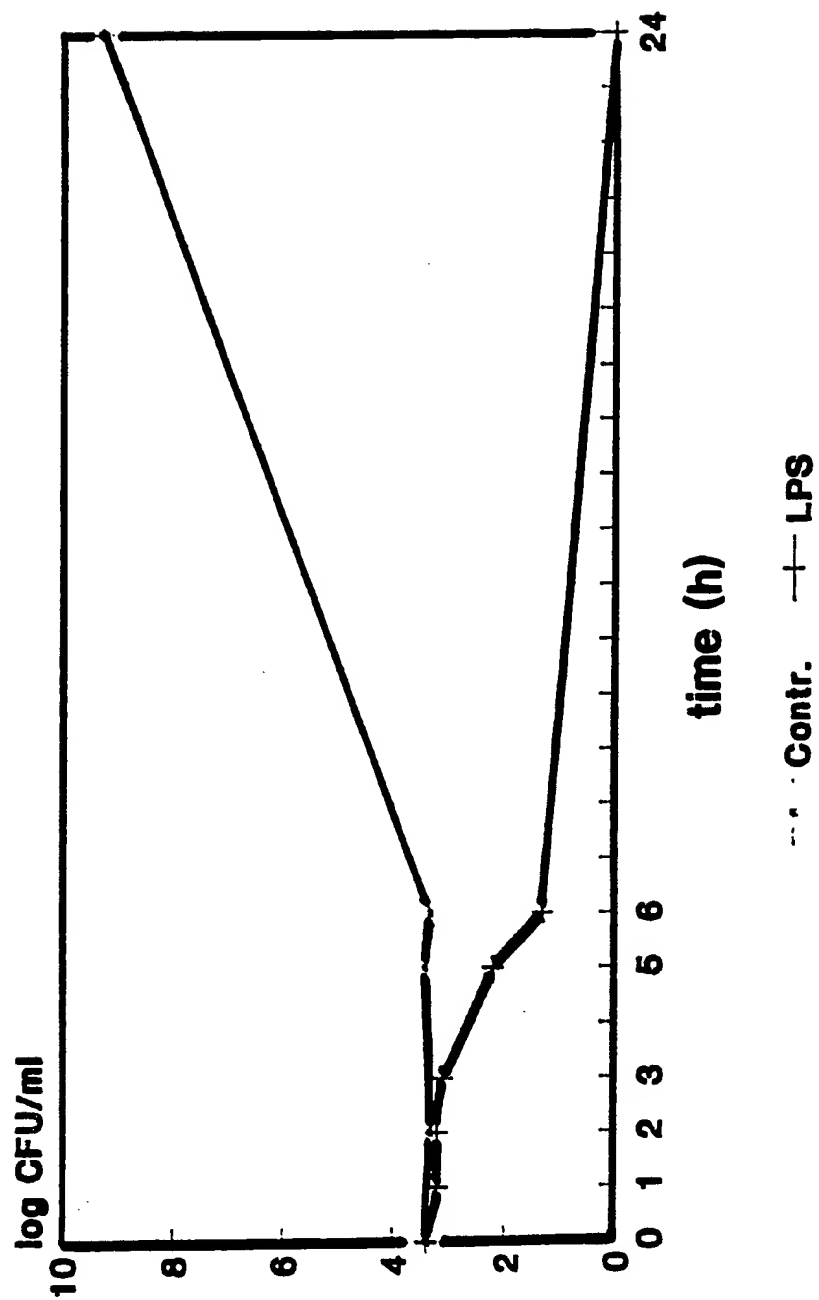


Figure 15

16/18

Effect LPS on *List.monocytogenes* Present on Camembert cheese

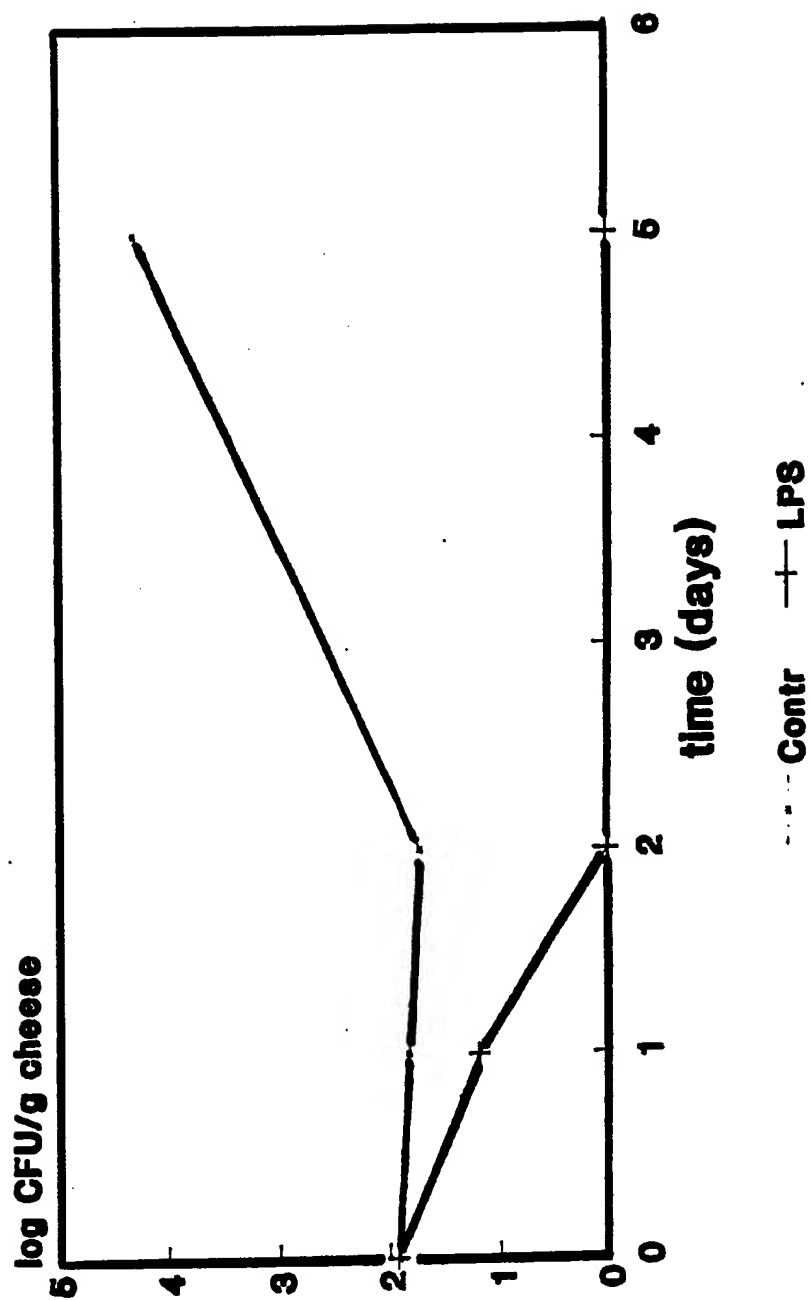


Figure 16

Production of d-gluconic acid

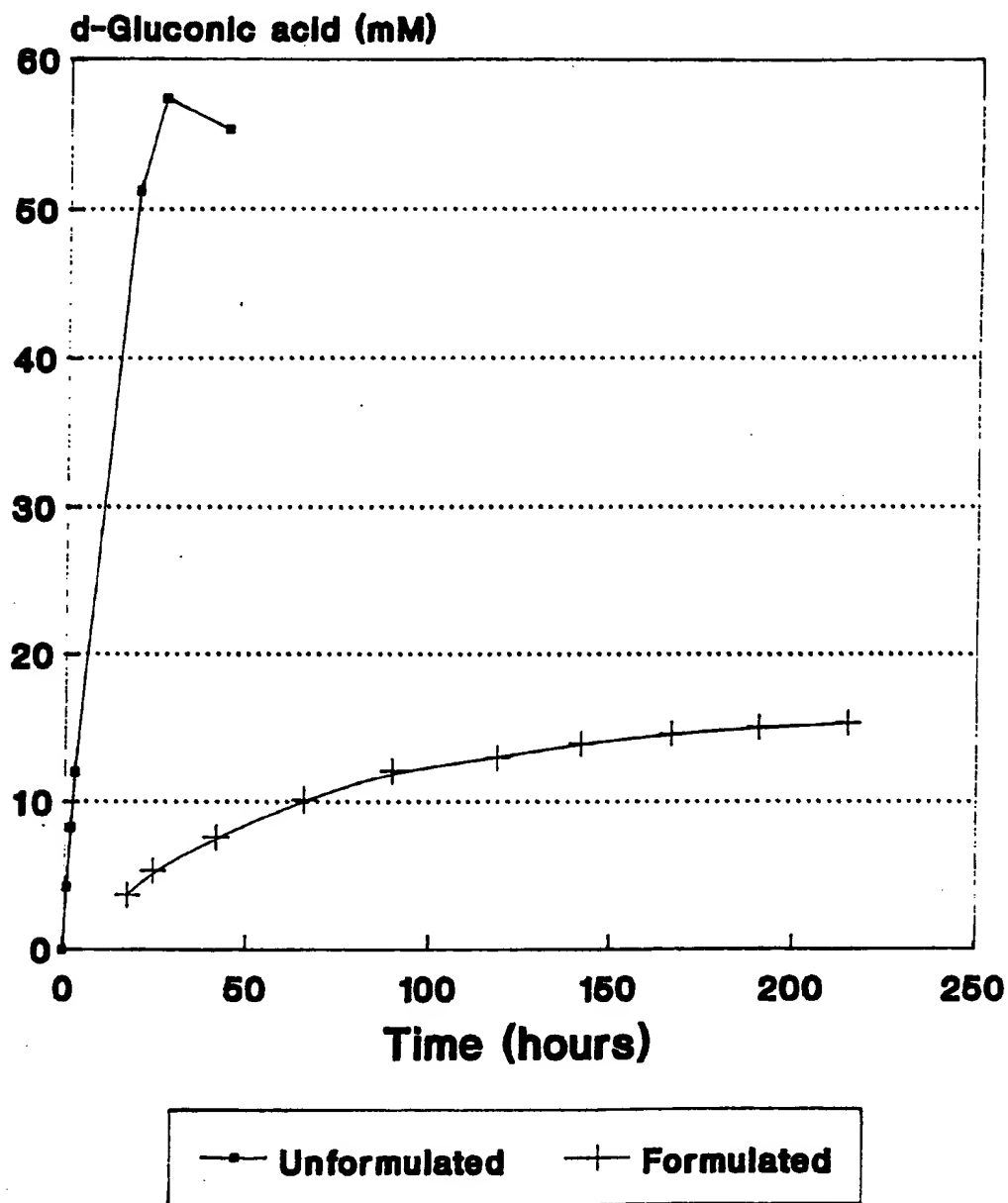


Figure 17

PRODUCTION OF GLUCONIC ACID

Product: 1 mg form./ml;
Buffer + 4.5 % NaCl pH 7.5; 7°C

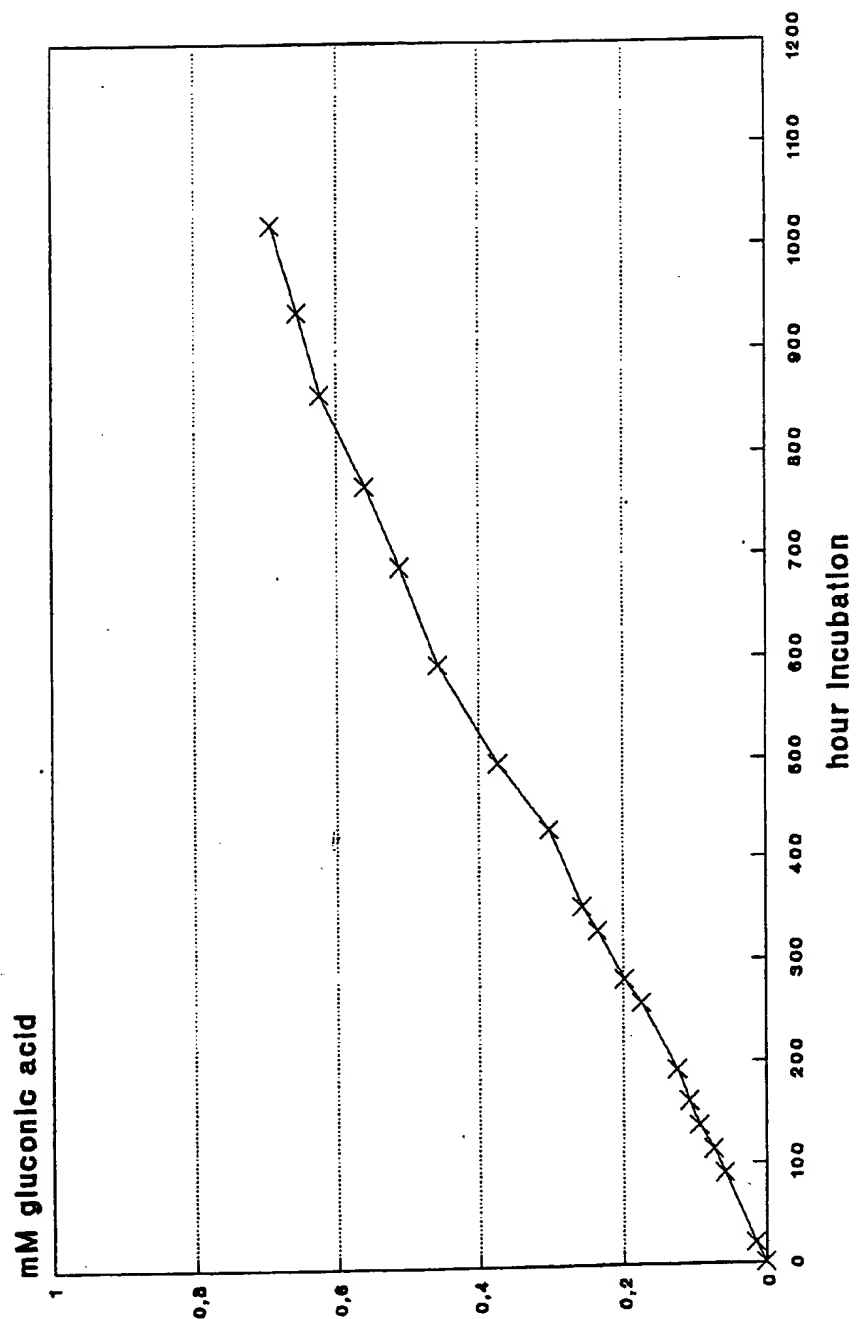


Figure 18

INTERNATIONAL SEARCH REPORT

International Application No

PCT/NL 92/00104

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A23L3/3571;	A23C19/032;	C12N11/02; A23C19/06
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A23L ; A23C	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	FR,A,2 237 589 (ALFA LAVAL AB) 14 February 1975 see the whole document ---	1,3-7; 9-14,17
X	EP,A,0 397 227 (BIO SERAE SA) 14 November 1990 see column 1, line 25 - column 2, line 14; claims 1,2,10	1,3,5,6, 11-14
Y	---	7-10
Y	EP,A,0 397 228 (BIO SERAE SA) 14 November 1990 see claims ---	7-10
A	FR,A,2 153 443 (GIST BROCADES NV) 4 May 1973 see claims 1,2,6 ---	2,16
-/-		
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search 01 OCTOBER 1992		Date of Mailing of this International Search Report 29.10.92
International Searching Authority EUROPEAN PATENT OFFICE		Signature of Authorized Officer LEPRETRE F.G.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	CHEMICAL ABSTRACTS, vol. 113, no. 24, 10 December 1990, Columbus, Ohio, US; abstract no. 214158h, see abstract & ANALYST vol. 115, no. 10, 1990, LONDON, GB pages 1289 - 1295 -----	13-15

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

NL 9200104
SA 61603

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 01/10/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A-2237589	14-02-75	SE-B- 375224	14-04-75
		AU-A- 6807974	23-10-75
		DE-A, C 2420135	06-02-75
		GB-A- 1468405	23-03-77
		JP-C- 1112651	16-09-82
		JP-A- 50069263	10-06-75
		JP-B- 56049535	24-11-81
		NL-A- 7405993	21-01-75
		SE-A- 7310076	20-01-75
EP-A-0397227	14-11-90	FR-A- 2646777	16-11-90
		CA-A- 2014847	12-11-90
		JP-A- 3193708	23-08-91
EP-A-0397228	14-11-90	FR-A- 2648321	21-12-90
		CA-A- 2014848	12-11-90
		JP-A- 3007537	14-01-91
		US-A- 5085873	04-02-92
FR-A-2153443	04-05-73	GB-A- 1404933	03-09-75
		AU-B- 466296	23-10-75
		AU-A- 4691672	28-03-74
		BE-A- 789195	22-03-73
		CA-A- 1020894	15-11-77
		CH-A- 612444	31-07-79
		DE-A, C 2246002	05-04-73
		NL-A- 7212445	27-03-73
		US-A- 3838007	24-09-74

EPO FORM PCT/92

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82